Ventrolateral medulla mechanisms involved in cardiorespiratory responses to central chemoreceptor activation in rats

Ana C. Takakura¹, Eduardo Colombari¹, José V. Menani¹ and Thiago S. Moreira².

1- Department of Physiology and Pathology, School of Dentistry, São Paulo State University (UNESP), 14801-903, Araraquara, SP, Brazil.
2- Department of Physiology and Biophysics, Institute of Biomedical Science, University of São Paulo (USP) 05508-900, São Paulo, SP, Brazil.

Running title: Ventrolateral medullary and central chemoreflex

Key words: chemoreflex, cardiorespiratory responses, ventrolateral medulla, sympathetic activity, phrenic nerve.

Number of text pages: 29
Number of tables: 1
Number of figures: 7

Address correspondence to:
Thiago S. Moreira, Ph.D.
Department of Physiology and Biophysics
Institute of Biomedical Science.
University of São Paulo
Av. Prof Lineu Prestes, 1524.
05508-900, São Paulo, SP, Brazil
Phone: +55 (11) 3091-7764
Fax : +55 (11) 3091-7285
E-mail: tmoreira@icb.usp.br

Acknowledgements
This research was supported by public funding from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (grant 06/60174-9 to TSM).
Abstract

A rise in arterial pCO2 stimulates breathing and sympathetic activity to the heart and blood vessels. In the present study, we investigated the involvement of the retrotrapezoid nucleus (RTN) and glutamatergic mechanisms in the Botzinger/C1 region (Botz/C1) in these responses. Splanchnic sympathetic nerve discharge (sSND) and phrenic nerve discharge (PND) were recorded in urethane anaesthetized, sino-aortic denervated, vagotomized and artificially ventilated rats subjected to hypercapnia (end-expiratory CO2 from 5% to 10%). Phrenic activity was absent at end-expiratory CO2 of 4%, and strongly increased when end-expiratory CO2 reached 10%. Hypercapnia also increased sSND by 103 ± 7%. Bilateral injections of the GABA-A agonist muscimol (2 mM) into the RTN eliminated the PND and blunted the sSND activation (Δ = +56 ± 8%) elicited by hypercapnia. Injections of NMDA receptor antagonist AP-5 (100 mM), non-NMDA receptor antagonist DNQX (100 mM) or metabotropic glutamate receptor antagonist MCPG (100 mM) bilaterally into the Botz/C1 reduced PND (Δ = +43 ± 7%, +52 ± 6% or +56 ± 11%, respectively). MCPG also reduced sSND (Δ = +41 ± 7%), whereas AP-5 and DNQX had no effect. In conclusion, the increase in sSND caused by hypercapnia depends on increased activity of the RTN and on metabotropic receptors in the Botz/C1, whereas PND depends on increased RTN activity and both ionotropic and metabotropic receptors in the Botz/C1.
**Introduction**

Hypercapnia stimulates the central and peripheral chemoreceptors, leading to the activation of breathing, as well as the activation of the sympathetic nervous system, leading to vasoconstriction and redirectioning of the cardiac output. In addition, CO$_2$ acts directly on the vasculature to cause vasodilation. In anesthetized, sino-aortic denervated and vagotomized rats, the vascular action of CO$_2$ dominates at the onset of hypercapnia, and the central mechanism dominates immediately after hypercapnia ends, leading to a transient hypertension (30).

Although the integration of cardiovascular and respiratory responses occurs at multiple levels of the central nervous system, the main mechanisms involved in the regulation of respiratory and sympathetic outflows are located in the ventrolateral medulla (12, 13, 17). Hypercapnia-induced sympathtoactivation is mediated to a large extent by the activation of presympathetic neurons in the rostral ventrolateral medulla (RVLM) henceforth called Botzinger/C1 region (Botz/C1), and occurs in bursts that are synchronized with phrenic nerve discharge (PND) (15, 24, 25, 28, 30). The central respiratory pattern generator and GABAergic mechanisms in the caudal ventrolateral medulla (CVLM) are important for the synchronization of the activity of presympathetic Botz/C1 neurons with respiratory activity, but not for the overall increase of activity of these neurons during CNS acidification (22, 26, 27, 30). Botz/C1 neurons may be activated directly by acidification *in vivo*, as well as by signals from various sources, including catecholaminergic neurons in the locus coeruleus, orexinergic neurons in the hypothalamus, A5 noradrenergic cells, serotonergic neurons in the raphe, and the cholinergic system located in the ventrolateral medulla. In addition, Botz/C1 presympathetic neurons may also
be activated by the retrotrapezoid nucleus (RTN) (1, 11, 17, 20, 23, 37). The RTN neurons are vigorously activated by hypercapnia *in vivo*, are uniformly activated by acidification *in vitro* and, appropriately, they remain CO₂-sensitive when the central respiratory pattern generator is experimentally silenced by morphine or blockade of glutamatergic synapses (1, 31, 32, 42). Furthermore, RTN neurons are glutamatergic and they directly innervate the Botz/C1 (1, 38).

Previous studies suggested that RTN neurons are important for hypercapnia-induced increase in breathing and showed that the blockade of glutamatergic ionotropic receptors in the Botz/C1 reduces hypercapnia-induced increase in PND and eliminates sympathoexcitation by peripheral chemoreceptor activation (15, 30, 32, 42). However, whether the same mechanisms are involved in hypercapnia-induced increase in sympathetic discharge has not been investigated. Therefore, we tested this hypothesis using anesthetized, vagotomized and artificially ventilated adult rats with or without denervated peripheral chemoreceptors to investigate whether RTN neurons and glutamatergic mechanisms within the Botz/C1 region contribute to the activation of SND produced by hypercapnia. In addition, the involvement of ionotropic or metabotropic glutamatergic receptors of the Botz/C1 region on hypercapnia-induced PND was also investigated.

**Materials and Methods**

**Animals**

Experiments were performed in 42 adult male Holtzman rats weighing 280-300 g. The experimental protocols were approved by the Animal Experimentation Ethics Committee of the Institute of Biomedical Science of the University of São Paulo.
**Surgery and anesthesia**

General anesthesia was induced with 5% halothane in 100% oxygen. Rats received a tracheostomy and then were artificially ventilated with 1.4-1.5% halothane in 100% oxygen until the end of the surgery. All rats were subjected to the following surgical procedures: femoral artery cannulation with polyethylene tubing (PE 10) for arterial pressure measurement, femoral vein cannulation for administration of fluids and drugs, removal of the occipital plate for insertion of a pipette for microinjections into the medulla oblongata via a dorsal transcerebellar approach, and skin incision over the lower jaw for placement of a bipolar stimulating electrode next to the mandibular branch of the facial nerve as previously described (30). The phrenic nerve was accessed by a dorsolateral approach after retraction of the right shoulder blade. All animals were bilaterally vagotomized to prevent any influence of artificial ventilation on PND. In most of the rats (n = 27) a complete baro- and peripheral chemoreceptor deafferentation was performed by sectioning the vagosympathetic trunks, the superior laryngeal nerves and the glossopharyngeal nerves (proximal to the junction with the carotid sinus nerves). In the other rats (baro- and chemoreceptor intact group, n = 15), the vagus nerves were carefully separated from the vagosympathetic trunk and selectively transected bilaterally. We presume that this procedure left the aortic depressor nerves intact but these very small nerves were not identified.

Splanchnic sympathetic nerve discharge (sSND) was recorded as previously described (30, 40). The right splanchnic nerve was isolated via a retroperitoneal approach, and the segment distal to the suprarenal ganglion was placed on a pair of teflon-coated silver wires that had been bared at the tip (250 µm bare diameter; A-M Systems, www.a-
msystems.com). The nerves and wires were embedded in adhesive material (Kwik-Cast Sealant, WPI, USA), and the wound was closed around the exiting recording wires.

Upon completion of surgical procedures, halothane was replaced by urethane (1.2 g/kg) administered slowly i.v. All rats were ventilated with 100% oxygen throughout the experiment. Rectal temperature (maintained at 37°C) and end tidal-CO₂ were monitored throughout the experiment with a capnometer (CWE, Inc, Ardmore, PA, USA) that was calibrated twice per experiment against a calibrated CO₂/N₂ mix. This instrument provided a reading of <0.1% CO₂ during inspiration in animals breathing 100% oxygen and an asymptotic, nearly horizontal reading during expiration. The adequacy of anesthesia was monitored during a 20 min stabilization period by testing for absence of withdrawal response, lack of AP change and lack of change in PND rate or amplitude to firm toe pinch. After these criteria were satisfied, the muscle relaxant pancuronium was administered at the initial dose of 1 mg/kg i.v. and the adequacy of anesthesia was thereafter gauged solely by the lack of increase in AP and PND rate or amplitude to firm toe pinch. Approximately hourly supplements of one-third of the initial dose of urethane were needed to satisfy these criteria during the course of the recording period (4 hours).

**In vivo recordings of physiological variables**

Mean arterial pressure (MAP), phrenic nerve discharge (PND), splanchnic nerve discharge (sSND), and tracheal CO₂ were recorded as previously described (30, 31, 40, 42).

The caudal and ventral boundaries of the facial motor nucleus were identified in each rat by the large (up to 5 mV) negative antidromic field potential generated in the facial motor nucleus by stimulating the mandibular branch of the facial nerve (for details see Ref. 6). Before starting the experiments, ventilation was adjusted to lower end-expiratory CO₂ to
4% at steady-state (60-80 cycles/min; tidal volume 1-1.2 ml/100 g). These conditions were
selected because 4% end-expiratory CO$_2$ was below the threshold of the PND.

All analog data (end-expiratory CO$_2$, sSND, PND and MAP) were stored on a
microcomputer via a micro-1401 digitizer from Cambridge Electronics Design (CED,
Cambridge, UK), and were processed off-line with Spike 2 software (version 6, CED).

Neural minute x volume (mvPND, a measure of the total phrenic nerve discharge
per unit of time) was determined by averaging iPND over 50 seconds and normalizing the
result by assigning a value of 0 to the dependent variable recorded at low levels of end-
expiratory CO$_2$ (below threshold) and a value of 1 at the highest level of end-expiratory
CO$_2$ investigated (between 9.5 and 10%). Stimulation of carotid chemoreceptors was
done with bolus injections of sodium cyanide (NaCN, 50 µg/kg, i.v.). Evidence that
cyanide acted on carotid chemoreceptors to activate brainstem neurons was obtained by
demonstrating that denervation of the carotid chemoreceptors eliminated the activation of
PND induced by cyanide (42).

**Intraparenchymal injections**

Drugs were bought from Sigma Chemicals Co. (St-Louis, MO, USA). They were
dissolved in sterile saline (pH 7.4) and pressure injected (30-50 nl in 5 s) bilaterally through
single-barrel glass pipettes (20 µm tip diameter). The GABA-A agonist muscimol was
diluted to 2 mM. The NMDA antagonist AP-5 (D,L-2-amino-5-phosphonovalerate), the
non-NMDA antagonist DNQX (6,7-dinitro-quinoxaline-2,3-dione), and the metabotropic
glutamate receptor antagonist MCPG [((+/-)-alpha-methyl-4-carboxyphenylglycine] were
diluted to 100 mM. All solutions contained a 5% dilution of fluorescent latex microbeads
(Lumafluor, New City, NY, USA) for subsequent histological identification of the injection sites and spread of the injections (31).

The glass pipettes containing the drug-microbead mixture also allowed recordings of field potentials used to direct the pipette tip to the desired sites. Injections into Botz/C1 were thus guided by recording the facial field potentials and were placed 200 μm ventral to the lower edge of the field, 300 μm caudal to the caudal end of the field and 1.8 mm lateral to the midline. Injections into the RTN were placed 250 μm ventral the lower edge of the facial field, 1.7 mm lateral to the midline and 200 μm rostral to the caudal end of the field. The electrophysiological recordings were made on one side only; the second injection was placed at the same coordinates on the contralateral side, 1-2 min later.

**Hypercapnia protocol**

Hypercapnia was produced by adding pure CO₂ to the oxygen supplied to the artificial ventilator to maintain end-expiratory CO₂ between 9.5 and 10%. This maximum end-expiratory CO₂ was maintained for 5 min; then the inspired gas was changed to 100% O₂. Each rat was subjected to three sessions of hypercapnia: 1) 10 min after bilateral injections of saline into the RTN or Botz/C1, 2) 10 min after bilateral injection of drug into the RTN or Botz/C1, and 3) 60 or 120 min after drug injection.

**Histology**

At the end of the experiments, rats were deeply anesthetized with halothane and perfused transcardially with saline followed by 10% buffered formalin (pH 7.4). The brain was removed and stored in fixative for 24 h at 4°C. The medulla was cut in 30 μm coronal sections with a vibrating microtome (Vibratome 1000S Plus, USA), and stored in a
cryoprotectant solution at -20°C (39). The injection site was verified with a conventional multifunction microscope (Olympus BX50F4, Japan). The most caudal section containing an identifiable cluster of facial motor neurons was identified in each brain and assigned a level of 11.6 mm caudal to bregma, as in the atlas of Paxinos and Watson (35). Coordinates of sections rostral and caudal of this reference section were calculated with respect to the reference section, using the number of intervening sections and the section thickness.

**Statistics**

Statistical analysis was done with Sigma Stat version 3.0 (Jandel Corporation, Point Richmond, CA). Data are reported as means ± standard error of the mean. One way ANOVA followed by the Newman-Keuls test was used for comparisons. Significance was set at p<0.05.

**Results**

**Histological analysis**

Typical injection sites in the RTN and Botz/C1 are shown in Figure 1. The beads included in the injectate spread approximately 260 μm on each side of the injection center. RTN injections centered 250 μm below the facial motor nucleus and 200 μm rostral to the caudal end of this nucleus (Fig. 1A), targeting the region that contains the highest density of CO₂-sensitive RTN neurons (31, 42). Botz/C1 injections were ventral to the nucleus ambiguus and approximately 300 μm caudal to the facial nucleus (Fig. 1B). Botz/C1 injection sites centered on the region that contains the bulk of the bulbospinal presympathetic neurons of the ventrolateral medulla (6, 39).
Cardiovascular, sympathetic and phrenic nerve responses to hypercapnia after bilateral injections of muscimol into the RTN

Hypercapnia (10% CO₂) in urethane anaesthetized, sino-aortic denervated and vagotomized rats caused an immediate hypotension (-19 ± 5 mmHg, n = 27) followed by a gradual return of MAP to control level, 30 to 40 s later. Immediately after hypercapnia ended, MAP increased by 35 ± 4 mmHg, and returned to control values 5 min later [F(1, 37) = 43.36, p<0.05] (Fig. 2A and 2B).

Bilateral injections of muscimol (2 mM, 30 nl each side, n = 7) into the RTN reduced baseline MAP (from 117 ± 2 to 99 ± 3 mmHg) [F(1, 10) = 27.04, p<0.05] and sSND by 26 ± 5% [F(1, 10) = 34.18, p<0.05]. Muscimol injections into the RTN had no effect in the hypotension produced by 10% CO₂ (Δ = -12 ± 3 mmHg, p>0.05), however, muscimol into the RTN reduced the increase in MAP produced at the end of the hypercapnia (Δ = +10 ± 6 mmHg) [F(1, 10) = 19.23, p<0.05] (Fig. 2A and 2B). Muscimol also reduced the hypercapnia-induced increase in sSND (Δ = +56 ± 8%) [F(1, 10) = 29.48, p<0.05], and abolished PND [F(1, 10) = 92.43, p<0.01] (Fig. 2A, 2C and 2D). The effect of muscimol was reversible: responses induced by hypercapnia returned to control within 2 hours (Fig. 2C and 2D).

Effects of muscimol injections in the RTN on responses induced by activation of peripheral chemoreceptors

In vagotomized rats with intact carotid sinus nerves (n = 6), bilateral injections of muscimol (2 mM, 30 nl each side) into the RTN reduced baseline MAP (from 115 ± 4 to 94 ± 6 mmHg), and reduced sSND by 22 ± 7%. Muscimol into the RTN reduced the rise in
arterial pressure (Δ = +26 ± 2, vs. saline: +37 ± 7 mmHg) [F(1, 8) = 26.43, p<0.05], sSND (Δ = 263 ± 21%, vs. saline: 387± 26%) [F(1, 8) = 37.13, p<0.05] and PND (Δ = +28 ± 5% of control baseline) [F(1, 8) = 112.64, p<0.01] produced by i.v injection of NaCN (50 μg/kg) (Fig. 3). In most of the rats (4/6), a reduced PND (Δ = +44 ± 7% of control baseline) could be transiently elicited by stimulating peripheral chemoreceptors with NaCN after muscimol injection into the RTN. In 2 of the 6 cases, muscimol into the RTN eliminated PND and no activity, even tonic, could be restored by peripheral chemoreceptors stimulation with NaCN (data not shown).

Cardiovascular, sympathetic and phrenic nerve responses to hypercapnia after bilateral injections of AP-5 or DNQX into the Botz/C1 region

Neither AP-5 (100 mM in 50 nl each side, n = 6) nor DNQX (100 mM in 50 nl each side, n = 6) bilaterally injected in the Botz/C1 region in sino-aortic denervated and vagotomized rats produced any effect on resting MAP (118 ± 5 or 116 ± 3 mmHg, respectively, vs. saline: 117 ± 4 mmHg) or sSND (106 ± 3% or 103 ± 5% of the control baseline).

The treatment with AP-5 or DNQX bilaterally into the Botz/C1 region did not modify the changes in MAP or sSND produced by hypercapnia, however, AP-5 [F(1, 8) = 15.93, p<0.05] or DNQX [F(1, 8) = 26.29, p<0.05] into the Botz/C1 region reduced PND induced by hypercapnia (Δ = +43 ± 7% and +52 ± 6%, respectively) (Figs. 4A-D and 5A-D). Hypercapnia-induced changes in PND returned to normal 60 min after AP-5 or DNQX injections (Fig. 4D and 5D).
Effects of AP-5 or DNQX injections into the Botz/C1 region on responses to peripheral chemoreceptor activation

Although injection of AP-5 and DNQX into the Botz/C1 region failed to reduce the sympathetic component of the central chemoreflex, a previous study showed that these treatments abolished the increase in MAP and ssND produced by activation of peripheral chemoreceptors (10, 41). Therefore, we tested the effects of the same doses of AP-5 and DNQX (100 mM in 50 nl) on the pressor and ssND responses to peripheral chemoreflex activation with i.v. NaCN.

In vagotomized rats with intact carotid sinus nerves (n = 4-5), bilateral injections of AP-5 or DNQX into Botz/C1 region did not change resting MAP (121 ± 5 or 118 ± 6, respectively, vs. control: 124 ± 6 mmHg) or resting ssND (98 ± 6, 99 ± 3% of the control baseline). However, as previously demonstrated, AP-5 [F(1, 5) = 24.83, p<0.05] or DNQX [F(1, 6) = 33.17, p<0.05] into the Botz/C1 region reduced the rise in arterial pressure (Δ = +11 ± 7 mmHg and +14 ± 4 mmHg, respectively, vs. saline: +32 ± 3 mmHg, p<0.05). Both AP-5 [F(1, 5) = 76.27, p<0.01] and DNQX [F(1, 6) = 65.17, p<0.01] also reduced ssND activation (Δ = +85 ± 8% and +108 ± 9%, respectively, vs. saline: 447± 38%, p<0.05) produced by i.v injection of NaCN (50 μg/kg) (Table 1).

Responses to hypercapnia after injection of MCPG into the Botz/C1 region

Bilateral injections of the metabotropic antagonist MCPG (100 mM in 50 nl in each Botz/C1 region, n = 8) in sino-aortic denervated and vagotomized rats did not alter resting MAP (119 ± 4 mmHg vs. saline: 121 ± 5 mmHg) and ssND (Δ = +103 ± 4% of control baseline). MCPG into the Botz/C1 region reduced the rise in MAP (Δ = +11 ± 5, vs. saline:
Δ = +25 ± 3 mmHg) [F(1, 13) = 16.42, p<0.05], sSND (Δ = +41 ± 7%) [F(1, 13) = 12.19, p<0.05] and PND (Δ = +56 ± 12%) [F(1, 13) = 11.73 p<0.05] to hypercapnia (Fig. 6A-D). MCPG had no effect on the initial hypotension produced by hypercapnia (Δ = -16 ± 4 vs. saline: Δ = -20 ± 3 mmHg, p>0.05) (Fig. 6A and 6B). These effects of MCPG were reversible: responses to hypercapnia returned to normal 60 min after MCPG injection (Fig. 6C and 6D).

Discussion

In vagotomized, sino-aortic baro- and chemoreceptor-denervated rats (completely denervated rats), under urethane anesthesia, increasing end-expiratory CO2 from 5 to 10% proportionally enhances PND and splanchnic SND (sSND) as a function of end-expiratory CO2 (30). Splanchnic SND exhibits a predominant peak activity coincident with early-inspiratory phase and the relationship between sSND or PND and end-expiratory CO2 was curvilinear, showing a threshold around end-expiratory 5% CO2 (30). In the present study, the changes in PND and sSND produced by hypercapnia (end-expiratory 10% CO2) were similar to those previously reported (30). The lack of sSND responses to acute changes in MAP produced by a pressor dose of phenylephrine or a depressor dose of sodium nitroprusside and the absence of pulse synchrony in the sSND confirm that the preparation was baro-denervated (data not shown, for more details, see Ref. 30).

Besides confirming that hypercapnia-induced changes in PND depend on RTN (42), the present results show that deactivation of RTN neurons with muscimol reduced hypercapnia-induced sympathoexcitation and pressor responses. In addition, the changes in PND, sympathoexcitation, and pressor responses to hypercapnia were dependent on glutamatergic mechanisms of the Botz/C1 region. However, whereas sympathoexcitation
and pressor responses depended on metabotropic receptors, PND depended on both metabotropic and previously demonstrated ionotropic receptors (15).

Although RTN and Botz/C1 region are adjacent nuclei, injection sites were separated by at least 500 μm. Given the small injection volumes (30 - 50 nl) and the limited spread of the fluorescent microbeads that were co-injected with the drugs (less than 300 μm from the injection site), it seems unlikely that the similarity of responses to injections in the RTN and the Botz/C1 region is caused by spread of the drugs between these areas.

**The retrotrapezoid nucleus contributes to the sympathoexcitation caused by central and peripheral chemoreceptor activation**

Bilateral injections of muscimol into the RTN eliminated PND and reduced the increase in sSND and arterial pressure produced by hypercapnia and by peripheral chemoreceptor activation, suggesting that both central and peripheral chemoreceptor-induced PND depend on RTN, as previously demonstrated (42, 43), whereas sympathoexcitation and pressor responses are only partially mediated by the RTN. Muscimol into the RTN also slightly reduced baseline arterial pressure and sSND, which suggests that sSND depends on tonic excitatory signals from the RTN. RTN neurons, considered one important region in the SNC that contains putative central chemoreceptors, innervates the Botz/C1 region (1, 31, 32, 38, 42, 43). Although the baseline activity of RTN neurons under the conditions of the present study is not enough to activate PND, it seems that baseline RTN activity is important for baseline sSND. Considering this possibility, an explanation for the reduced sympathoexcitation elicited by hypercapnia or cyanide, after muscimol into the RTN, might also be the decrease in the excitability of presympathetic neurons after removing baseline facilitatory signals from the RTN and not the reduction of
chemoreceptor signals to these neurons. In this case, signals to increase sSND during hypercapnia would arise from other central areas, except RTN. Therefore, the involvement of RTN on chemoreceptor-induced increases in sSND might be related to the existence of RTN baseline facilitatory signals to presympathetic neurons not dependent on CO₂ or to facilitatory signals produced by a direct action of CO₂ in the RTN (or signals that arise from peripheral chemoreceptors to RTN). With the present results, both possibilities are still open.

Although injection of muscimol into the RTN reduced sympathoexcitation induced by hypercapnia, it did not increase the hypotension induced by hypercapnia. Perhaps a reason for the maintenance of hypotension at the same level was the reduced baseline sympathetic activity and arterial pressure, resulting in less efficient CO₂-induced vasodilation under these conditions.

**Rostral ventrolateral medulla as main relay of central and peripheral sympathetic chemoreflexes**

Hypercapnia markedly increases sSND and the activity of presympathetic neurons in the Botz/C1 region (18, 19, 29, 30, 36). The present results suggest that these effects are mediated by glutamatergic metabotropic receptors in the Botz/C1 region, and that ionotrophic receptors in the Botz/C1 region are not involved in these responses. However, the present and previous studies showed that ionotropic glutamatergic receptor blockade in the Botz/C1 region eliminates sympathoexcitation to peripheral chemoreceptor activation (15, 21, present results). The effect of NMDA or non-NMDA receptor blockade in the Botz/C1 region on peripheral chemoreflex is consistent with anatomical and electrophysiological evidence that suggests that signals from peripheral chemoreceptors
reach the Botz/C1 region and the RTN by direct glutamatergic projections from the commissural nucleus of the solitary tract (2, 16, 41, 42). According to the present results, central sympathetic chemoreflex was reduced by metabotropic receptor blockade, not by ionotropic glutamatergic antagonists into the Botz/C1 region, which suggests that central and peripheral chemoreflexes do not share the same mechanisms to produce sympathetic activation.

Although Botz/C1 region neurons might be directly activated by CO₂, the effect of CO₂ on Botz/C1 neurons, in vivo, is likely mediated by synaptic inputs to the Botz/C1 region arising from pH sensitive neurons. The RTN neurons that innervate Botz/C1 region are pH sensitive, express VGLUT2 (an indication that they release glutamate), and their inactivation with muscimol reduces sympathoexcitation and pressor responses induced by hypercapnia and cyanide (32, 38, 42, present results). Therefore RTN neurons may be an important source for glutamatergic facilitatory signals to Botz/C1 region during hypercapnia and during activation of peripheral chemoreceptors. Even if the increase in sSND and pressor response to hypercapnia are the result of a direct action of CO₂ on Botz/C1 neurons, these effects are at least partially dependent on metabotropic receptor activation in the Botz/C1 region and on RTN neuron activity as suggested by the reduction of these responses by muscimol into the RTN and MCPG into the Botz/C1 region. The glutamatergic metabotropic antagonist MCPG used in the present study is classified as a competitive antagonist more selective for mGluR1 and mGluR5 (5, 7). Metabotropic receptor activation in the Botz/C1 region was previously shown to increase arterial pressure and stimulate respiration (33, 44). In the present study, only one dose of MGCP was tested and, therefore, is not possible to know if increasing the blockade of metabotropic receptors
in the Botz/C1 region with a higher dose of MGCP would result in a more effective reduction of hypercapnia-induced sympathoexcitation and pressor response.

**Alternative sources of central chemoreceptor input to the sympathetic outflow**

Muscimol into the RTN only partially reduced the hypercapnia-induced increase in sSND, which suggests that only part of the signals produced by hypercapnia to increase sSND arise from the RTN. Therefore, either Botz/C1 neurons are CO₂ sensitive, or the signals that activate the Botz/C1 region arise from other areas. Some NTS neurons are CO₂ sensitive *in vitro* (8) and focal acidification of the NTS by dialysis of a high CO₂ solution stimulates breathing *in vivo* (34), suggesting that the NTS neurons may have chemoreceptor properties. Wake-ON orexinergic and noradrenergic neurons also contribute to the “waking neural drive to breathe”, the neural mechanism that maintains breathing during waking regardless of the CO₂ level (4, 11). These wake-ON neurons are activated by high levels of hypercapnia and target virtually all sites involved in generating the sympathetic tone (9, 11) suggesting that they may contribute to the sympathetic component of the central chemoreflex. Pontine noradrenergic neurons also display a modest degree of pH sensitivity *in vitro* and, similar to serotonergic neurons located in the midline raphe, are activated by hypercapnia and can contribute to sSND activation (20, 37).

It has also been suggested that astrocytes are an important component of the central mechanism involved in autonomic regulation (3). In addition, there is increasing evidence that adenosine-triphosphate (ATP), possibly released by astrocytes, is an important mediator of central chemoreception (Gourine et al., 2005). *In vivo* studies showed that hypercapnia caused a discrete release of ATP in the RTN (14). This might influence RTN
neurons that signal to increase SND through metabotropic receptors within the Botz/C1 region.

**Involvement of glutamatergic mechanisms in phrenic nerve activity**

A previous study showed that blockade of glutamatergic ionotropic receptors in the Botz/C1 region with kynurenic acid reduces hypercapnia-induced increases in PND (30). The present results confirm the involvement of ionotropic receptors and also show the involvement of metabotropic receptors in the Botz/C1 region in this response. Therefore, both ionotropic and metabotropic receptors in the Botz/C1 region are part of the mechanisms activated by CO₂ to increase PND. Metabotropic receptors in the Botz/C1 region are also involved in hypercapnia-induced sympathoexcitation and pressor responses, which suggests that respiratory and cardiovascular responses to hypercapnia share, at least partially, the same mechanisms in the Botz/C1 region. Ionotropic receptors in the Botz/C1 region are involved in hypercapnia-induced increases in PND, but not in cardiovascular responses, which is similar to the effects reported for ionotropic receptors in the RTN (30).

**Conclusion**

Figure 7 is a schematic model of the possible medullary mechanisms involved in cardio-respiratory responses to chemoreceptor activation based on the present and previous results (22, 30, 42). Signals from central or peripheral chemoreceptors may directly or indirectly affect the activity of various medullary areas, including the RTN, NTS, Botz/C1 region and CPG, which in turn control sympathetic discharge to heart and blood vessels and motoneuron discharge to respiratory muscles. An essential step for hypercapnia-induced PND is the activation of RTN neurons by CO₂/H⁺, which in turn send facilitatory signals to
activate the CPG, either directly or through activation of metabotropic and ionotropic glutamate receptors in the Botz/C1 region. Signals from the RTN that activate metabotropic receptors in the Botz/C1 region may also increase sympathetic activity to the cardiovascular system. It is possible that CO$_2$/H$^+$ sensors outside the RTN also contribute to activation of sSND during hypercapnia.

**Perspectives**

The present results show for the first time that in urethane anesthetized rats the increase in sSND produced by hypercapnia is partially dependent on activity of RTN neurons, whereas Botz/C1 region metabotropic receptor activation is involved in hypercapnia-induced increase in sSND and PND. They also confirm the importance of RTN neurons and ionotropic receptors in the Botz/C1 region for the increase in PND during hypercapnia. Although the results show the importance of glutamatergic metabotropic mechanisms in the Botz/C1 region for hypercapnia-induced sympathoexcitation, it is still not clear if this response depends totally on Botz/C1 region mechanisms or that other central areas are also involved. The same is true for the source of signals that activate Botz/C1 neurons and sSND during hypercapnia. Whereas injection of muscimol in the RTN completely blocked hypercapnia-induced PND, it only blunted hypercapnia-induced sSND, which suggests that signals driving sympathetic activation may arise from other sources, including the presympathetic neurons in the Botz/C1 region. It is important also to consider that the present results are from anesthetized rats and it is well known that anesthetic may affects centrally mediated cardiorespiratory responses. Therefore, more studies are necessary to improve the understanding of the central mechanisms involved on
hypercapnia-induced cardiorespiratory responses, including tests to confirm if similar conclusions would apply also to conscious rats.
Table 1: Effects of injection of AP-5 or DNQX in the Botz/C1 region on peripheral chemoreflex stimulation with sodium cyanide (NaCN, 50 μg/kg).

<table>
<thead>
<tr>
<th></th>
<th>MAP (mmHg)</th>
<th>SND (%)</th>
<th>Number of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (saline)</td>
<td>32 ± 3</td>
<td>447 ± 38</td>
<td>9</td>
</tr>
<tr>
<td>AP-5</td>
<td>11 ± 7*</td>
<td>85 ± 8*</td>
<td>5</td>
</tr>
<tr>
<td>DNQX</td>
<td>14 ± 4*</td>
<td>108 ± 9*</td>
<td>4</td>
</tr>
</tbody>
</table>

Values are mean ± SE. * Significantly different from control (saline). MAP, mean arterial pressure; NaCN, sodium cyanide; SND, sympathetic nerve discharge.


10. de Paula PM, Branco LG. Glutamatergic receptors of the rostral ventrolateral medulla are involved in the ventilatory response to hypoxia. Respir Physiol Neurobiol. 146, 125-34, 2005.


Figure legends

Figure 1: Injection sites in the ventrolateral medulla.
(A) Photomicrograph of a coronal section showing bilateral injections in the RTN. Bregma = -11.3 mm. (B) Coronal section showing bilateral injections in the Botz/C1 region. Bregma = -11.96 mm. Arrows indicate injection sites. IO, inferior olive; nA, nucleus ambiguus; py, pyramide; Sp5, spinal trigeminal tract; VII, facial motor nucleus. Scale bar is 1 mm.

Figure 2: Muscimol injections into the retrotrapezoid nucleus (RTN) attenuate the effect of hypercapnia on sSND in vago-sino-aortic denervated rats.
(A) Recordings from one rat showing the effect of injection of muscimol into the RTN on changes in arterial pressure (AP), splanchnic sympathetic nerve discharge (sSND), and phrenic nerve discharge (PND), induced by an increase of end-expiratory CO₂ from 5 to 10%. Responses were recorded 10 min after bilateral injection of saline into the RTN, and 10 min after bilateral injection of muscimol (2 mM, 30 nl each side) in the RTN. (B) Changes in mean arterial pressure (ΔMAP), (C) sSND (ΔsSND) and (D) PND (ΔmvPND) elicited by stepping end-expiratory CO₂ from 5 to 10% during saline or muscimol injections into the RTN. Differences expressed as a percentage of the response to the CO₂ challenge elicited during saline injection. (E) sites of injections. Rec: recovery period (>120 min after muscimol injections). mvPND = minute * volume PND. *different from saline (p<0.05); n = 7 rats.
Figure 3: Muscimol injections into the retrotrapezoid nucleus (RTN) attenuate the cardiorespiratory effect of peripheral chemoreceptor activation.

(A) Changes in mean arterial pressure (ΔMAP), (B) sSND (ΔsSND) and (C) PND (ΔmvPND) elicited by i.v. sodium cyanide (NaCN, 50 μg/kg) after bilateral injections of saline or muscimol into the RTN. *different from saline (p<0.05); n = 6 rats.

Figure 4: AP-5 injections into the Botzinger/C1 region (Botz/C1 region) do not change the effect of hypercapnia on sSND in vago-sino-aortic denervated rats.

(A) Recordings from one rat showing the effect of injection of AP-5 into the Botz/C1 region on changes in arterial pressure (AP), splanchnic sympathetic nerve discharge (sSND), and phrenic nerve discharge (PND), induced by an increase of end-expiratory CO₂ from 5 to 10%. Responses were recorded 10 min after bilateral injection of saline into the Botz/C1 region, and 10 min after bilateral injection of AP-5 (100 mM, 50 nl each side) in the Botz/C1 region. (B) Changes in mean arterial pressure (ΔMAP), (C) sSND (ΔsSND) and (D) PND (ΔmvPND) elicited by stepping end-expiratory CO₂ from 5 to 10% during saline or AP-5 injections into Botz/C1 region. Difference expressed as a percentage of the response to the CO₂ challenge elicited during saline injection. (E) injection sites. Rec: recovery period (60 min after AP-5 injections). mvPND = minute * volume PND. *different from saline (p<0.05); n = 6 rats.

Figure 5: DNQX injections into the Botzinger/C1 region (Botz/C1 region) do not change the effect of hypercapnia on sSND in vago-sino-aortic denervated rats.
(A) Recordings from one rat showing the effect of injection of DNQX into the Botz/C1 region on changes in arterial pressure (AP), splanchnic sympathetic nerve discharge (sSND), and phrenic nerve discharge (PND), induced by an increase of end-expiratory CO₂ from 5 to 10%. Responses were recorded 10 min after bilateral injection of saline into the Botz/C1 region, and 10 min after bilateral injection of DNQX (100 mM, 50 nl each side) in the Botz/C1 region. (B) Changes in mean arterial pressure ($\Delta$MAP), (C) sSND ($\Delta$sSND) and (D) PND ($\Delta$mvPND) elicited by stepping end-expiratory CO₂ from 5 to 10% during saline or DNQX injections into Botz/C1 region. Difference expressed as a percentage of the response to the CO₂ challenge elicited during saline injection. (E) injection sites. Rec: recovery period (60 min after DNQX injections). mvPND = minute * volume PND. *different from saline (p<0.05); n = 6 rats.

Figure 6: MCPG injections into the Botzinger/C1 region (Botz/C1 region) attenuate the effect of hypercapnia on sSND in vago-sino-aortic denervated rats.

(A) Recordings from one rat showing the effect of injection of MCPG into the Botz/C1 region on changes in arterial pressure (AP), splanchnic sympathetic nerve discharge (sSND), and phrenic nerve discharge (PND), induced by an increase of end-expiratory CO₂ from 5 to 10%. Responses were recorded 10 min after bilateral injection of saline into the Botz/C1 region, and 10 min after bilateral injection of MCPG (100 mM, 50 nl each side) in the Botz/C1 region. (B) Changes in mean arterial pressure ($\Delta$MAP), (C) sSND ($\Delta$sSND) and (D) PND ($\Delta$mvPND) elicited by stepping end-expiratory CO₂ from 5 to 10% during saline or MCPG injections into Botz/C1 region. Difference expressed as a percentage of the response to the CO₂ challenge elicited during saline injection. (E) injection sites. Rec:
recovery period (60 min after MCPG injections). \( \text{mvPND} = \text{minute} \times \text{volume PND} \).

\*different from saline \( p<0.05 \); \( n = 8 \) rats.

**Figure 7: Role of the ventrolateral medulla on sympathetic chemoreflex**

Schematic model of the possible medullary mechanisms involved in the control of cardiorespiratory responses caused by raising cerebral pCO\(_2\). Signals from central or peripheral chemoreceptors may directly or indirectly affect the activity of several medullary areas, including the RTN, NTS, Botz/C1 region, and CPG, which affect sympathetic discharge to heart and blood vessels and motorneurons to respiratory muscles. An essential step for hypercapnia-induced PND is activation of RTN neurons by CO\(_2\)/H\(^+\), which in turn send excitatory signals to activate the CPG, either directly or through activation of metabotropic and ionotropic glutamate receptors in the Botz/C1 region. Signals from the RTN that activate metabotropic receptors in the Botz/C1 region may also increase sympathetic activity to the cardiovascular system. Abbreviations: CPG, central pattern generator; commNTS, commissural nucleus of the solitary tract; IgLuR, glutamatergic ionotropic receptors; MgLuR, glutamatergic metabotropic receptors; RTN, retrotapezoid nucleus; Botz/C1, Botzinger/C1 region; SPGn, sympathetic preganglionic neurons.
A

CO2 (%)

AP (mmHg)

sSND (a.u.)

iPND (a.u.)

Saline

MCPG

5 min

B

ΔMAP (mmHg)

Saline  MCPG

C

ΔsSND (%)

Saline  MCPG  Rec

D

ΔmvPND (%)

Saline  MCPG  Rec

E

Diagram of brain regions: Amb, IO, Py, Sp5.